Diagnostic molecular pathology, part 2: Proteomics and clinical applications of molecular diagnostics in hematopathology

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Currently, molecular diagnostics plays an important role in the clinical management of a wide array of hematologic neoplasms, solid tumors, infectious diseases, and inherited genetic disorders. In this second part of our series, we conclude our review of esoteric techniques by summarizing some of the advances in the field of "proteomics." We then turn our discussion to the role of molecular technology in the diagnosis, prognostication, and clinical management of lymphoid and leukemic neoplasms. The article also addresses the crucial role of molecular diagnostics in monitoring engraftment status following marrow transplantation.

PROTEOMICS

The term *proteomics*, coined in 1995, indicates a large-scale characterization of the entire protein complement of a cell type, tissue, or organism. By no means, however, should the discipline of proteomics be considered a novelty. In fact, two-dimensional gel electrophoresis was developed over 20 years ago and has been in use in routine clinical laboratory assays such as fetal lung maturity tests. Two-dimensional gel electrophoresis offers a high-resolution capacity to separate and characterize proteins in complex mixtures. Although two-dimensional gel electrophoresis remains an important tool for protein identification, it is now being coupled with mass spectrometry analysis as a means to characterize complex milieus under study (1, 2).

It is becoming more and more evident that the hypothesis of "one gene encodes one protein" is no longer valid. As shown in *Figure 1*, alternative splicing, posttranslational modifications, compartmentalization, and translocalization allow for many protein isoforms to be coded by a single gene allele. It then follows that appropriate integration of genomic and proteomic data is crucial to elucidate protein functions as they relate to pathogenesis *(Figure 2)* (2). By focusing on the study of genetic abnormalities, researchers may miss the opportunity to discover potentially important deviations at the protein level (epigenetics) that can be as rewarding to diagnosis and therapy. Integration of genomics and proteomics will no doubt facilitate discovery of novel drug target proteins and biomarkers of diseases (3).

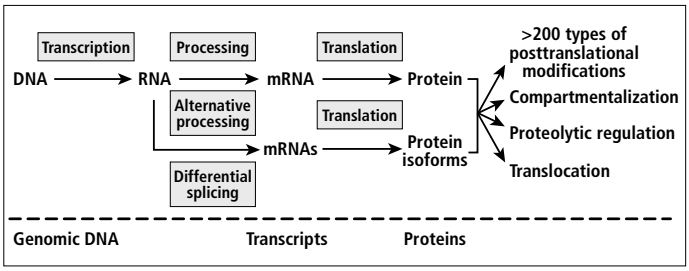

Figure 1. Different epigenetic processes that may lead to physically and/or functionally variable forms of protein isoforms from a single gene sequence. Reprinted with permission from reference 2.

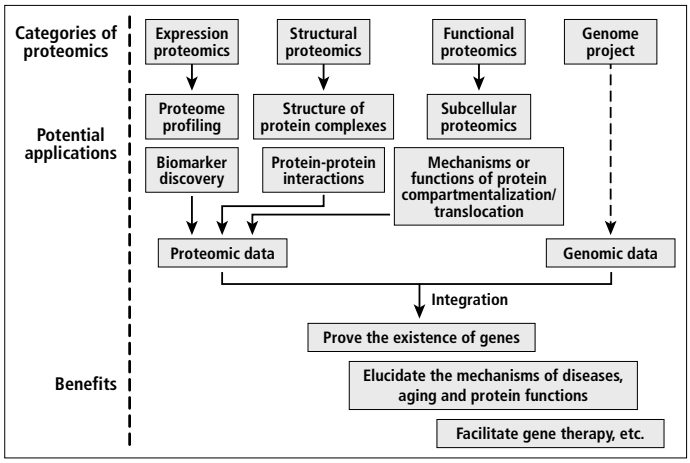

Figure 2. Proteomics and genomics integration. The integration of data will have a synergistic effect on biologic discovery. Reprinted with permission from reference 2.

Mass spectrometry

Several mass spectrometry platforms are commercially available; we focus our discussion on surface-enhanced laser desorption/ionization (SELDI) technology (4–6). SELDI uses matrix-assisted laser desorption and time-of-flight (TOF) analysis to allow fully automated separation of even minute amounts of protein components. The assay exploits variations in mass and electric charge of the different protein components in a given sample (e.g., serum).

Using SELDI-TOF technology coupled with ProteinChip systems and arrays (Ciphergen Biosystems, Fremont, California), femtomole amounts of thousands of proteins can be resolved di-

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Table 1. Results of a sentinel ovarian carcinoma proteomic study showing excellent sensitivity and specificity for serum mass spectrometry analysis*

	Classification by proteomic pattern		
	Cancer	Unaffected	New cluster
Unaffected women			
No evidence of ovarian cysts	2/24	22/24	0/24
Benign ovarian cysts <2.5 cm	1/19	18/19	0/19
Benign ovarian cysts >2.5 cm	0/6	6/6	0/6
Benign gynecological disease	0/10	1/10	9/10
Nongynecological inflammatory disorder	0/7	0/7	7/7
Women with ovarian cancer			
Stage I	18/18	0/18	0/18
Stage II, III, IV	32/32	0/32	0/32

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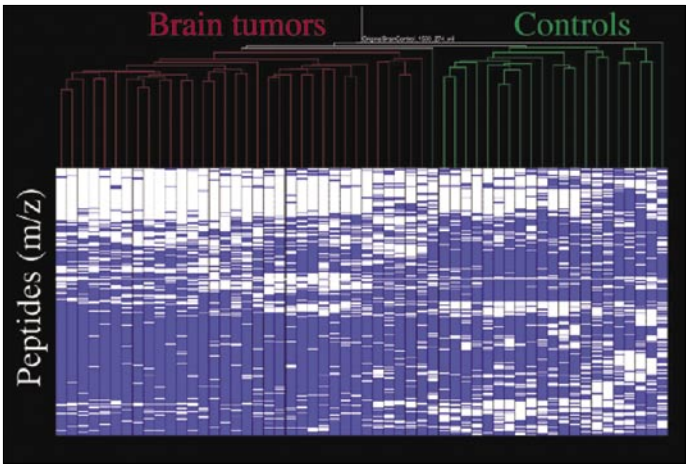

Figure 3. Proteomic fingerprints of the sera of 32 patients with glioblastoma multiforme compared with 22 controls using mass spectrometry. Expression patterns of 274 informative peptides are depicted using hierarchical clustering. Reprinted with permission from reference 10. Copyright 2004 American Chemical Society.

rectly from as little as a microliter of serum (7). Furthermore, archival air-dried cytologic smears obtained by fine-needle aspiration cytology can be analyzed, as shown by Fetsch et al (8). The sample is applied to a chip containing up to 24 distinct regions on an aluminum support (i.e., the ProteinChip array). The aluminum surface is coated according to interest with one of a variety of substances that have a desired chemical or electrical characteristic (hydrophobic vs hydrophilic, cationic vs anionic). Alternatively, the chip surface could be coated with a specific antibody or protein receptor targeting a protein group of interest. These coated affinity surfaces capture proteins on the basis of their physical, electrochemical, or antigenic qualities.

The chip is then exposed to an ultraviolet nitrogen laser beam that dislodges the captured protein (desorption), transforming it into a gaseous ion (ionization) by protonization. The gaseous ions are accelerated in an electric field and "fly" to hit a detector. It is the variation in TOF that allows for separation of the different protein components originally captured on the chip surface. The TOF variation is based on the mass/charge ratio of each peptide. Peptides with a higher mass take a longer time to

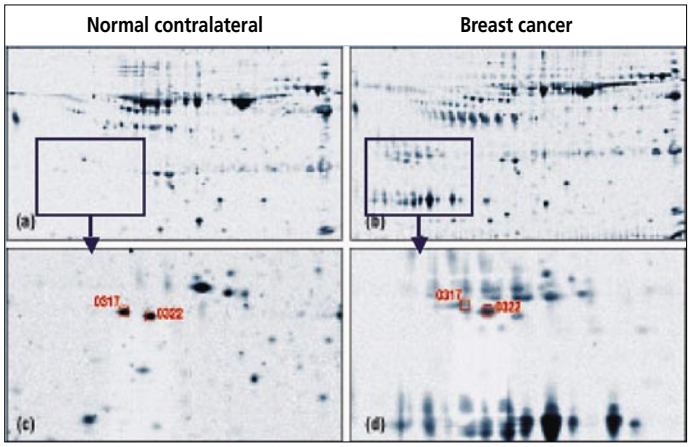

Figure 4. Proteomic analysis of nipple aspirate fluids using two-dimensional gel electrophoresis. Spots in the bottom panel (enlarged from the top panel) indicate proteins expressed solely by nipple aspirate fluids from breasts containing tumor and not by the contralateral control breast. Copyright 2002 American Cancer Society. Reprinted from reference 11 by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

fly to the detector. With the aid of computer analysis, results are displayed as peaks representing the different peptide components of a given sample.

By characterizing proteomic "fingerprints" associated with a particular type of tumor, it is possible to identify an unknown tumor sample that matches a previously defined fingerprint. This can be done on the basis of the "peaks" pattern even without actually knowing which protein each peak represents.

Expressional proteomics

Among the several disciplines already emerging within the field of proteomics, expressional proteomics could lend itself most readily to molecular diagnostic applications. In 2002, a National Institutes of Health group led by a pathologist, Lance Liotta, MD, published their results of what many now consider the sentinel study in the field of expressional proteomics (9). The objective of their study was to identify a serum proteomic pattern that would help in the early detection of ovarian carcinoma. Given the fact that currently available serologic markers such as CA125 fail to detect up to 50% of cases of early stage ovarian carcinoma, a highly sensitive, noninvasive screening assay will be invaluable for such a devastating disease.

Using data obtained by mass spectrometry analysis of 100 samples, a "training set" composed of 50 serum samples from patients with benign ovarian cysts and 50 samples from ovarian carcinoma patients, the National Institutes of Health group was able to develop an algorithm of differential proteomic patterns for the two groups. They next tested their algorithm on a masked 116-sample testing set composed of 50 serum samples from ovarian carcinoma patients and 66 controls. Their astonishing results are summarized in *Table 1*. The newly developed proteomic-based algorithm was able to correctly identify all 50 patients with ovarian carcinoma, including all 18 patients with stage I disease.

Several similar studies have followed, illustrating the great potential of proteomic analysis for early detection of neoplasia. In a study conducted at Memorial Sloan-Kettering Cancer Center, Villanueva et al (10) were able to demonstrate a proteomic fingerprint pattern for glioblastoma multiforme using serum

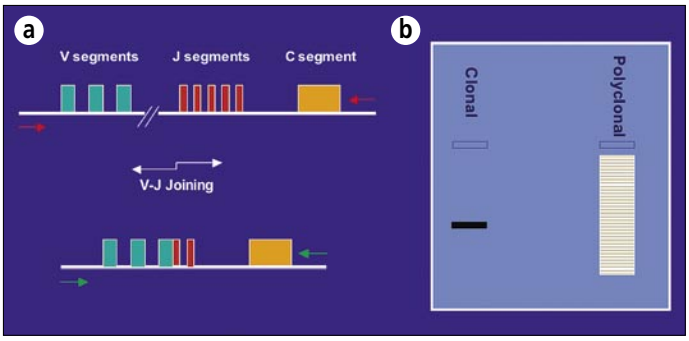

Figure 5. (a) Schematic depiction of immunoglobulin heavy-chain gene rearrangement studies by polymerase chain reaction. **(b)** Results of agarose gel electrophoresis. A solid black (clonal) band indicates a positive reaction, whereas the smear of faint bands on the right indicates a reactive inflammatory polyclonal process.

mass spectrometry analysis followed by hierarchical clustering data analysis *(Figure 3)* similar to that widely used in DNA chip technology. The latter is based on "clustering" cases according to similarity in proteomic (or genomic) fingerprint pattern, resulting in a branched-tree–like depiction in which most similar cases are listed in neighboring branches. In two other intriguing studies, REF, the proteomic breast cancer "fingerprint," was identified in nipple aspiration fluids analyzed by two-dimensional gel electrophoresis (11) *(Figure 4)* or SELDI-TOF mass spectrometry (4).

CLINICAL APPLICATIONS OF MOLECULAR DIAGNOSTICS IN HEMATOPATHOLOGY

Immunoglobulin heavy-chain gene and T-cell receptor gene rearrangement

In the appropriate setting, gene rearrangement studies are a powerful tool to establish a diagnosis of lymphoma. The test can be invaluable by establishing evidence of monoclonality in a morphologically suspect lymphoid infiltrate.

Currently, gene rearrangement studies are most commonly performed using a polymerase chain reaction (PCR)-based assay that exploits physiologic genetic rearrangements (deletions) occurring during B- and T-cell lymphocyte differentiation in response to antigen stimulation. As illustrated in *Figure 5a*, when B lymphocytes undergo rearrangement of their immunoglobulin heavy-chain (IgH) gene, portions of the gene sequence are deleted, leading to formation of a shorter new sequence that can be flanked by a set of consensus PCR primers (complementary nucleic acid sequences) that allow for amplification of the new sequence. A neoplastic (clonal) lymphocytic population shares an IgH gene sequence identical in size and composition. This leads to a PCR product homogenous in both size and electric charge that electrophoretically migrates as a single band when run on an agarose gel *(Figure 5b)*. The PCR product migrates as a single peak when analyzed using a capillary electrophoresis–equipped sequencing instrument *(Figure 6)*. This assay gives today's pathologist a powerful tool that can add an additional level of diagnostic confidence when faced with a biopsy (fresh or paraffin-embedded archival) containing morphologically suspect lymphoid cells.

However, the need to integrate clinical data with findings on histology, flow cytometry, and gene rearrangement studies cannot be overemphasized. Documenting clonality does not always equate with a diagnosis of malignancy. Certain reactive inflammatory conditions such as autoimmune processes and infectious conditions (e.g., hepatitis C, *Helicobacter pylori*) (12) can contain a clonal B- or T-cell population detectable by gene rearrangement studies. Finally, it is wise to remember that the sensitivity of a gene rearrangement assay can vary from 40% to 80% depending on the technique used by a specific laboratory (13).

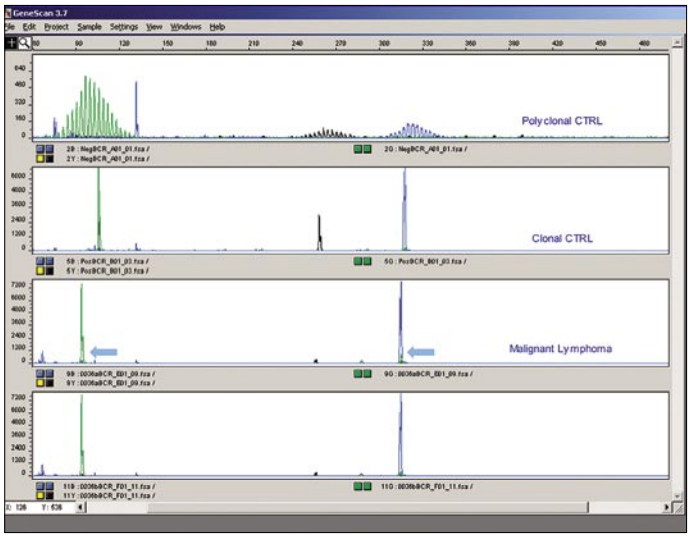

Figure 6. Results of capillary electrophoresis of the immunoglobulin heavy-chain gene rearrangement polymerase chain reaction assay. The third row (arrows) and fourth row illustrate clonal peaks similar to the clonal control (second row), supporting a diagnosis of malignant B-cell lymphoma in this paraffin-embedded sample from a lymph node biopsy.

Molecular cytogenetics

Molecular cytogenetics is defined as the characterization of chromosomal abnormalities using molecular techniques such as PCR and reverse-transcription PCR (RT-PCR), Southern blot, or fluorescence in situ hybridization (FISH). FISH offers the added advantage of being applicable to both metaphase and interphase targets while preserving crucial morphologic and topographic findings. These techniques lend themselves to identification of chromosomal translocations, deletions, inversions, and numerical aberrations.

The essential role now played by molecular cytogenetics in the diagnosis, monitoring, and management of chronic myelogenous leukemia (CML) offers a great paradigm for future integration of molecular diagnostics in the management of other neoplastic diseases.

The characteristic translocation of CML, i.e., *BCR-ABL*/t(9;22)(q34;q11.2), results in the formation of a chimeric (fusion) gene (*BCR-ABL*, or the Philadelphia chromosome) with a tyrosine kinase function continuously promoting proliferation in affected leukocytes (14). The *BCR-ABL* mRNA transcripts can be detected and quantified in blood samples of new patients by using real-time RT-PCR. The latter not only helps establish the diagnosis of CML but offers a baseline measurement for future monitoring of the patient's response to targeted therapy with imatinib mesylate (Gleevec). *BCR-ABL* translocation can also be detected by FISH (15). Several types of probes are available for that purpose, as shown in *Figure 7*. Furthermore, FISH analysis for other markers in CML, such as trisomy 8 *(Figure 8a)* (16, 17) and chromosome 9q34 deletion (18, 19) *(Figure 8b)*, can help

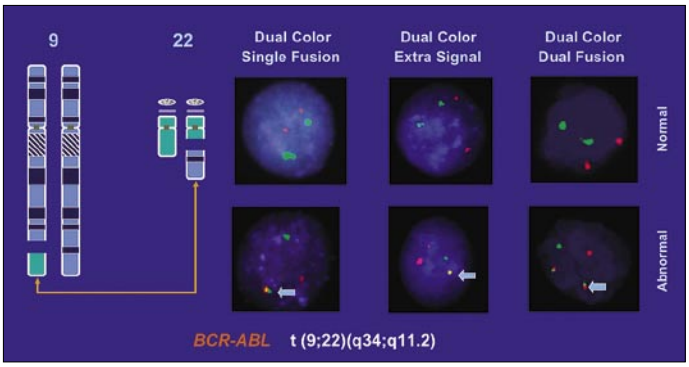

Figure 7. Fluorescence in situ hybridization analysis for *BCR-ABL* chromosomal translocations. A yellow signal in columns 1 and 2 and a green-red fusion signal in column 3 indicate a positive test. Modified with permission from Abbott Diagnostics.

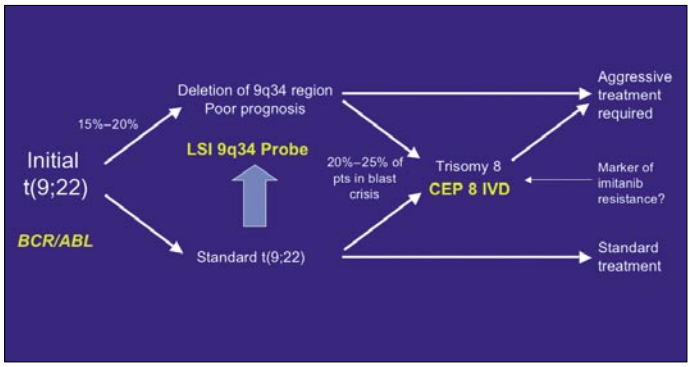

Figure 9. Schematic of the utility of fluorescence in situ hybridization in the treatment and prognostication of chronic myelogenous leukemia. Modified with permission from Abbott Diagnostics.

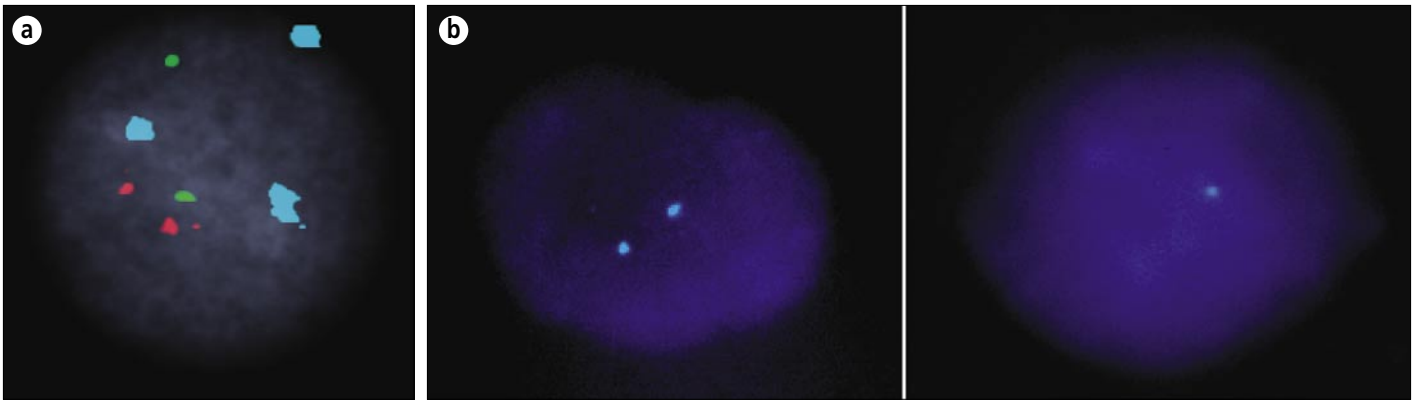

Figure 8. Fluorescence in situ hybridization prognostic markers in chronic myelogenous leukemia. **(a)** A nucleus showing trisomy 8 (3 aqua), while *BCR-ABL* ES fusion is negative (two green; two red). Reprinted from reference 17 with permission from the International Society for Experimental Hematology. **(b)** Fluorescence in situ hybridization LSI 9q34. The normal cell (left) shows two intact copies of chromosome 9 (two aqua signals), and the abnormal cell (right) shows loss of one of the 9q34 signals. Reprinted with permission from Abbott Diagnostics.

Table 2. Widely used molecular cytogenetic tests for diagnosis, prognostication, and monitoring of hematologic neoplasms

Disease	Test	Assay	Clinical utility	Comment
CML	Philadelphia chrom/t(9;22)	FISH	Diagnosis	
CML	Philadelphia chrom/t(9;22)	Q-RT-PCR	Diagnosis; target of imatinib therapy and monitoring of response	Quantitative measurement of mRNA (MRD)
CML	Trisomy 8	FISH	Poor prognosis: blast crisis	Clonal evolution after imatinib therapy
CML	Del9q34	FISH	Poor prognosis; resistance to imatinib	
CLL	Trisomy 12	FISH	Poor prognosis	(ref 20)
CLL	Del13q14	FISH	Better prognosis	(ref 20)
ALL	Tel-AML1/t(12;22)	FISH	Better prognosis (ref 21)	Cryptic (not detectable) by routine cytogenetics
ALL	Philadelphia chrom/t(9;22)	Q-RT-PCR or FISH	Worse prognosis, MRD, target of imatinib therapy	(refs 22, 23)
FCL	Bcl2-IgH/t(14;18)	PCR or FISH	Diagnosis	
AML M4	INV 16	Q-RT-PCR or FISH	Diagnosis	Quantitative mRNA, can be cryptic (ref 24)
AML M5	PML-RARA/t(15;17)	Q-RT-PCR	Diagnosis; predict response to ATRA therapy (ref 25)	Quantitative mRNA
MZL	Bcl1-IgH/t(11;14)	PCR or FISH	Diagnosis	

CML indicates chronic myelogenous leukemia; chrom, chromosome; FISH, fluorescence in situ hybridization; Q-RT-PCR, real-time reverse-transcriptase polymerase chain reaction; MRD, minimal residual disease; CLL, chronic lymphocytic lymphoma; ALL, acute lymphocytic lymphoma; FCL, follicular cell lymphoma; AML, acute myelogenous leukemia; INV, inversion; ATRA, all-trans-retinoic acid; MZL, mantle zone lymphoma.

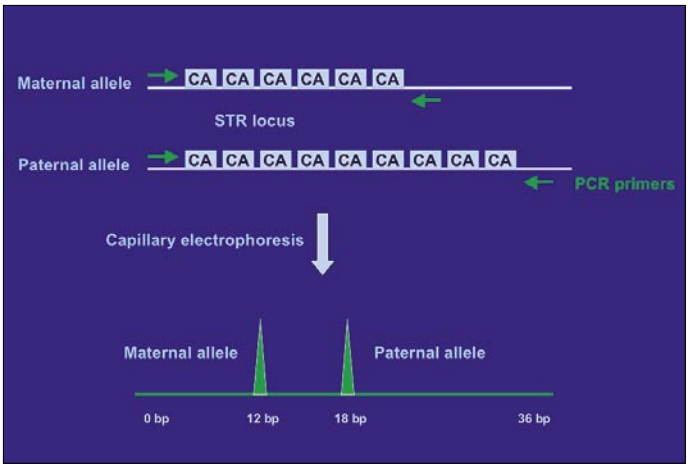

Figure 10. Schematic of short tandem repeat markers. In this heterozygous example, differences in the number of CA repeats (6 vs 9) between the two alleles result in two distinct polymerase chain reaction peaks by capillary electrophoresis. These peaks can be used as genetic markers of this individual identity.

Figure 11. Evaluation of engraftment by short tandem repeat analysis after bone marrow transplantation. Peripheral blood leukocyte subpopulations were analyzed (rows 3, 4, 5, 6). Arrows indicate informative peaks that distinguish donor and recipient identity; these peaks can be useful in evaluating the source of leukocytes in blood samples after transplantation.

detect clonal evolution and the need for more aggressive therapy *(Figure 9)*.

A rapidly growing number of assays are now offered to help manage several hematologic neoplasms. *Table 2* includes some that are more commonly used. These tests are of great clinical utility in helping to predict prognosis (e.g., trisomy 12 in chronic lymphocytic leukemia), select appropriate therapy (e.g., promyelocytic leukemia–retinoic acid receptor α [PML-RARA] in acute myelogenous leukemia–M5), or monitor response to treatment (*BCR-ABL*/t(9:22) in CML). Molecular cytogenetic testing has the advantage of a faster turnaround time and at times superior sensitivity compared with conventional cell culture and G-banding–based cytogenetic tests. Tel-AML1 translocation in acute lymphoblastic leukemia and inversion 16 in acute myelogenous leukemia–M4Eo are examples of "cryptic" abnormalities that could not be detected by conventional cytogenetic techniques (20, 24).

CHIMERISM ENGRAFTMENT STUDIES

Chimerism analysis after bone marrow or stem cell transplantation offers an objective, quantitative measure for monitoring engraftment of donor cell populations in the new host. Engraftment assays exploit differences in genetic markers between donor and recipient cells. Short tandem repeats (STR), or microsatellites, are noncoding DNA loci, each composed of repeated units of 2 to 7 nucleotides (usually dinucleotides or trinucleotides). STR are polymorphous markers that vary in the number of repeats within a specific STR locus. Each individual inherits one paternal and one maternal allele of a specific STR locus, leading to further diversity in terms of his or her STR pattern *(Figure 10)*.

PCR analysis of STR loci highlights differences among pretransplant donor and recipient leukocytes. Loci that are different between donor and recipient are considered informative and are subsequently monitored after transplantation to determine engraftment status *(Figure 11)* (26–29). Recently, posttransplantation chimerism analysis has been used in the assessment of minimal residual disease (MRD). Since STR analysis by PCR has a sensitivity of only 1% to 5% for the minor population in a chimeric sample, an additional step of cell sorting is performed that specifically selects for the neoplastic (e.g., leukemic) cells; this increases the sensitivity of the STR assay, thus allowing evaluation of MRD after bone marrow transplantation. Early detection of the presence of MRD allows for early intervention strategies, such as decreased immunosuppression or donor lymphocyte infusion, that target the neoplastic population (graft-vs-leukemia effect) (27, 28).

Finally, (X,Y) FISH analysis can be used to evaluate engraftment in cases of gender-mismatch transplantation (27). This technique is based on the labeling (by painting or centromeric probes) of the X and Y chromosomes in leukocytes after bone marrow transplantation. Cells can thus be easily assigned a donor or recipient genetic origin (27). (X,Y) FISH–based chimerism analysis can be instrumental in cases of across-gender bone marrow transplant in which pretransplantation donor or recipient samples are not readily available for STR analysis.

CONCLUSION

Proteomics holds great promise in providing pathologists and clinicians with new sensitive, noninvasive tools for early detection of neoplasms such as ovarian carcinoma. The application of molecular diagnostics in hematologic neoplasms is a model for its application in the wider arena of solid tumors. Currently, gene rearrangement studies, molecular cytogenetics, and chimerism analysis are providing pathologists and oncologists with highly accurate tools to successfully navigate the difficult diagnostic and management pathways in hemato-oncology.

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